INHIBITION AND AGING OF CHANNEL CATFISH BRAIN ACETYLCHOLINESTERASE FOLLOWING EXPOSURE TO TWO PHOSPHOROTHIONATE INSECTICIDES AND THEIR ACTIVE METABOLITES

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*The inhibition and aging of acetylcholinesterase (AChE) in fingerling channel catfish (*Ictalurus punctatus*) brain tissue was studied after single in vivo exposures to high levels of chlorpyrifos (0.25 mg/L), chlorpyrifos-oxon (7 μg/L), parathion (2.5 mg/L), or paraoxon (30 μg/L). Exposure to both parent compounds produced identical initial inhibition (95%), but in the later sampling times there was significantly more inhibited AChE in the chlorpyrifos-treated fish than in the parathion-treated fish (47% and 28%, respectively, on d 16). There were higher levels of aged AChE following chlorpyrifos exposure than following parathion exposure, but differences were not significant. Exposure to both oxons produced initial inhibition greater than 90%, and patterns of recovery and aging were statistically similar between both compounds; no significant inhibition was observed after d 11. The similar patterns of inhibition, recovery, and aging between the two oxon treatments, which have similar lipophilicities, suggest that the greater amount of AChE inhibition and aging observed in the chlorpyrifos-treated fish compared with the parathion-treated fish probably results from the higher lipophilicity of chlorpyrifos than of parathion. Overall, the prolonged brain AChE inhibition exhibited in catfish exposed to phosphorothionates is not the result of aging of the inhibited enzyme but is the result of either a slow rate or a lack of spontaneous reactivation.*

The commercial production of channel catfish, *Ictalurus punctatus*, has increased dramatically in recent years (Tucker & Robinson, 1990). Because of the high toxicity of the synthetic pyrethroid insecticides to fish, organophosphorus (OP) insecticides have become the compounds of choice for application to agricultural fields adjacent to aquaculture ponds. In this situation,

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overspray by agricultural applicators could easily result in contamination of these ponds with OPs. Additionally, the possibility of overspray or runoff from agricultural fields could result in toxicity to the endemic organisms in natural bodies of water.

Parathion and chlorpyrifos are phosphorothionate insecticides that are toxic to many species of fish including channel catfish (Mayer & Ellersieck, 1986). There is about a 10-fold difference in toxicity between the two compounds in 1 g channel catfish, with LC50 values of 0.28 and 2.65 mg/L reported for chlorpyrifos and parathion, respectively. In fish, acetylcholinesterase (AChE) is considered to be the direct target for these compounds and other OPs (Weiss, 1958; Benke et al., 1974; Jarvinen et al., 1983; van der Wel & Welling, 1989). Following absorption, these phosphorothionate insecticides are bioactivated to their active metabolites, the oxons, which rapidly inhibit brain AChE. This inhibition is maintained for several weeks in fish (Benke & Murphy, 1974; Coppage et al., 1975), and has been observed with chlorpyrifos (van der Wel & Welling, 1989; Boone, 1991) and parathion (Benke & Murphy, 1974; Boone, 1991). The mechanisms behind this persistent inhibition are not well understood. One possible explanation is a slow rate of reactivation of the phosphorylated enzyme, which could delay the return of enzyme activity to control levels. However, another possibility could be the subsequent "aging" of AChE following inhibition by the active metabolite. Aging of phosphorylated AChE is the poorly understood process of dealkylation rendering the inhibited enzyme refractory to reactivation (Wilson et al., 1992). If phosphorylated fish brain AChE ages rapidly, this process could contribute to the overall toxicity of OP insecticides by preventing spontaneous recovery of the enzyme.

Inhibition and aging of AChE by OPs depends on the interaction of the inhibitor with the enzyme. The inhibitor initially associates with the anionic site of AChE, which orients the inhibitor such that phosphorylation of the active site can occur. Once phosphorylation occurs, the portion of inhibitor that is associated with the anionic site, the "leaving group," is cleaved. Thus, the anionic site of AChE may function to orient the inhibitor such that association with the nucleophilic active site is preferable for some inhibitors compared with others. Such a situation appears to exist between the active metabolites of chlorpyrifos and parathion, with chlorpyrifos-oxon being a much more potent in vitro inhibitor of catfish brain AChE than paraoxon (Straus & Chambers, 1992). Following phosphorylation of AChE by either paraoxon or chlorpyrifos-oxon and departure of their respective leaving groups, the moiety that remains bound to the enzyme is theoretically identical between these inhibitors. This suggests that the phosphorylated enzymes are identical and therefore should behave the same. However, since a large difference in inhibitory potency exists between the two compounds, there could be differences in the steric orientation of the moiety within the active site, even though chemically identical. In fact, differences in the aging and reactivation of rat brain AChE inhibited by the nerve gas soman and its thio-

choline-like analog have been reported even though the resulting inhibited enzymes were chemically identical (Bošković et al., 1968). Spontaneous reactivation of AChE was much greater with the analog than with soman; aging was greater with soman. Thus, differences in the structure of the leaving group can result in differences in the steric orientation of the moiety remaining attached to the enzyme. If that positioning is such that the dealkylation step is favored with one of the compounds, the amount of aged AChE would be greater with it. The greater ability of one compound to age would decrease the amount of AChE that could be reactivated and hence lead to more persistent inhibition and possibly greater toxicity. Since chlorpyrifos is much more toxic to channel catfish than is parathion (Mayer & Ellersieck, 1986), the higher affinity of chlorpyrifos-oxon than paraoxon for AChE could enhance aging and therefore contribute to the greater toxicity of the parent compound. However, it is not known what role aging plays, if any, in the impact of an OP insecticide on channel catfish or in the overall determination of the toxicity of the insecticide to that species.

The investigation of aging of AChE following exposure to anticholinesterases has been generally restricted to studies involving nerve gases in mammalian systems, with limited literature available on aging of AChE following exposure to OP insecticides, especially in nontarget species such as catfish. These experiments were designed to elucidate any differences in the aging of phosphorylated AChE from catfish brain following in vivo exposure to chlorpyrifos and parathion. To eliminate any confounding factors such as lipophilicity or bioactivation differences between the two parent compounds, the active metabolites paraoxon and chlorpyrifos-oxon, which have similar lipophilicity, were also tested.

METHODS

Chemicals

Analytical grade parathion, paraoxon, chlorpyrifos, and chlorpyrifos-oxon were synthesized in the Department of Entomology, Mississippi State University, by Dr. Howard Chambers, as previously described (Chambers & Chambers, 1989). Biochemicals were obtained from Sigma Chemical Company (St. Louis, Mo.).

Experimental Conditions

Channel catfish fingerlings weighing 30–70 g were obtained from the College of Veterinary Medicine, Mississippi State University, Mississippi State, Miss. Fish were placed into 68-L silicone-bound glass aquaria at an initial stocking rate of 20 fish/aquarium. Each aquarium contained 60 L of dechlorinated municipal (Starkville, Miss.) tap water (pH 7.5–7.7 and an alkalinity 60–90 mg/L as $CaCO_3$) with a constant flow of 1 L/min such that total water exchange occurred within 1 h. Water temperature was main-

tained at 30°C throughout experimentation. The fish were acclimated for 2 wk prior to initiation of treatments. The fish were maintained on a 12 : 12 light–dark cycle with constant aeration and were fed the minimum amount of commercial crumbled catfish feed daily to maintain body weight.

Treatment of Animals

Before treatment, the fish were not fed for 48 h. The phosphorothionates were tested at nominal concentrations of 2.5 mg/L parathion and 0.25 mg/L chlorpyrifos, and the active metabolites at nominal concentrations of 30 μg/L paraoxon and 7 μg/L chlorpyrifos-oxon. The toxicants were dissolved in 25 ml acetone for each aquarium, and the control tanks received the same amount of acetone. The concentrations of compounds administered were determined as the maximum amount that would achieve greater than 90% inhibition of brain AChE but would not produce excessive mortality.

At initiation of treatment, water flow through the tanks was stopped, the toxicants were added, and the water was gently stirred to insure even distribution. The fish were exposed to the toxicants for 4 h in a static environment, after which water flow was restored at 1 L/min. The fish were not fed during this 4-h static toxicant exposure period. Dissolved oxygen was monitored during this static period.

In both experiments, there were three aquaria for the controls and treatments, with each aquarium representing a single replication. All compounds were added at time 0, and a single catfish was removed from each of the control and treated aquaria at 4, 8, 12, and 16 h, and 1, 2, 4, 6, 8, 10, 12, 14, and 16 d following initiation of exposure. The fish were euthanitized by decapitation. The brains were rapidly removed and frozen at –70°C until enzyme assays were conducted.

Biochemical Analysis

Each whole brain was homogenized in cold 0.05 *M* Tris-HCl buffer (pH 7.4), and the AChE activity and the amount of aged AChE present were immediately determined simultaneously for each sample. The assay temperature was 30°C.

AChE activity was measured spectrophotometrically using a modification (Chambers et al., 1988) of Ellman et al. (1961), using acetylthiocholine as the substrate and 5,5′-dithiobis(2-nitrobenzoate) as the chromogen. Duplicate subsamples were run for all samples at a final concentration of 0.2125 mg wet weight/ml.

The amount of aged AChE was estimated by in vitro incubation of parallel inhibited samples in the presence or absence of 0.5 m*M* TMB-4 (an oxime reactivator), similar to methods described previously (Chambers & Chambers, 1989). Reactivation proceeded in concentrated homogenate that was diluted 40-fold prior to assay of AChE activity to reduce the concentration of TMB-4. Preliminary in vitro experiments to test the efficacy of TMB-4 as a reactivator of inhibited catfish brain AChE demonstrated that incubation of inhibited

AChE with TMB-4 for 30 min resulted in greater than 95% reactivation. For each homogenate, the specific activity obtained from the initial discontinuous AChE assay was compared with the corresponding vehicle (water) incubated specific activity, and no loss or gain of activity was detected during the 30-min incubation.

For each sampling time, the mean specific activity from the TMB-4-incubated homogenates of the control group and the mean specific activities from TMB-4-incubated homogenates of the treated groups were used to calculate the percentage of total AChE that was aged. Likewise, the mean specific activity from the vehicle-incubated homogenates of the control group and the mean specific activities from vehicle-incubated homogenates of the treated groups were used to calculate the percentage of total AChE that was inhibited. If the percent aged AChE was equal to or larger than the percent inhibited AChE within the same treatment in the same sampling time, all of the inhibited AChE was considered to be aged.

Protein was quantified with the Folin phenol reagent using bovine serum albumin as a standard (Lowry et al., 1951). AChE specific activity was calculated as nanomoles product formed per minute-milligram protein.

Statistics

Statistical analysis was performed using SAS on a personal computer. For the vehicle-incubated homogenates, the inhibited and control AChE specific activities were analyzed by GLM followed by separation of means by LSMEANS. Similar analysis was performed on the TMB-4 incubated homogenates. A level of $p \leq .05$ was used to conclude a significant difference between treated and control means within each sampling time and significant differences among means within each treatment.

RESULTS

During administration of the OP compounds, the dissolved oxygen in the aquaria remained high (above 2.5 mg/L) and the fish were not exposed to low oxygen conditions. The catfish exhibited signs of distress including lethargy and difficulty in maintaining their position in the water column with all compounds except chlorpyrifos. Occasional mortality was observed in the chlorpyrifos and parathion treatments. The observable signs of toxicity decreased with time and no visible effects were evident by d 2.

To give an indication of sample variability and significant changes in specific activity over time within each treatment group during the phosphorothionate exposures, the specific activity means, SEM, and statistical results for selected times are presented in Table 1. The calculated percent inhibited and aged AChE from the means in Table 1 and from all additional sampling times are shown in Figure 1. The data from the active metabolite exposures are displayed similarly in Table 2 and Figure 2.

Following in vivo treatment with parathion and chlorpyrifos, the AChE

TABLE 1. Specific Activities[a] of Inhibited and Aged Acetylcholinesterase from the Brain of Catfish Exposed to 2.5 mg/L Parathion or 0.25 mg/L Chlorpyrifos In Vivo

Time	Control for vehicle[b]	Control for TMB-4[c]	Chlorpyrifos		Parathion	
			Vehicle	TMB-4	Vehicle	TMB-4
4 h	467.8 ± 35.9^{A}	454.0 ± 16.7^{A}	28.2 ± 9.3^{A}	364.6 ± 34.6^{A}	27.0 ± 20.4^{A}	341.1 ± 17.8^{A}
2 d	458.9 ± 27.1^{A}	417.2 ± 16.8^{A}	98.1 ± 6.4^{AB}	277.4 ± 20.5^{AB}	126.5 ± 11.8^{B}	300.5 ± 20.2^{A}
4 d	449.2 ± 4.4^{A}	403.5 ± 26.1^{A}	86.7 ± 16.6^{AB}	236.8 ± 12.2^{BC}	146.0 ± 8.1^{BC}	251.1 ± 28.7^{A}
8 d	466.3 ± 28.9^{A}	459.7 ± 15.1^{A}	127.6 ± 18.3^{BC}	228.0 ± 11.9^{C}	202.9 ± 22.3^{C}	284.1 ± 42.8^{A}
12 d	455.8 ± 26.6^{A}	431.1 ± 71.4^{A}	157.6 ± 21.3^{C}	237.0 ± 21.3^{BC}	294.2 ± 27.7^{D}	293.4 ± 52.5^{A}
16 d	501.3 ± 34.8^{A}	464.4 ± 17.9^{A}	263.4 ± 41.1^{D}	283.9 ± 18.5^{ABC}	363.4 ± 30.3^{E}	341.7 ± 17.1^{A}

[a]Specific activity is expressed as nmol product formed/min-mg protein, mean ± SEM of three catfish. Means within each column not followed by the same capital letter are significantly different ($p \leq .05$). All means from chlorpyrifos or parathion exposures are significantly different from controls ($p \leq .05$).

[b]Specific activities were obtained from brain homogenates preincubated with the reactivator vehicle. Differences from control specific activities represent amount of inhibited AChE.

[c]Specific activities were obtained from brain homogenates preincubated with the reactivator, TMB-4. Differences from control specific activities represent amount of aged AChE.

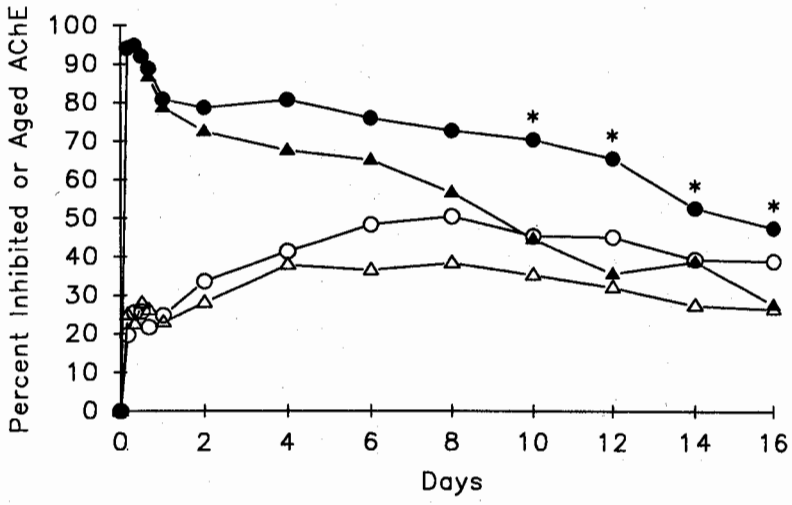

FIGURE 1. Plot of percent inhibited (closed symbols) and aged (open symbols) acetylcholinesterase in catfish brain as a function of time following a 4-h in vivo exposure to 2.5 mg/L parathion (triangles) or 0.25 mg/L chlorpyrifos (circles). Asterisks indicate statistically significant differences ($p \leq .05$) in the amount of inhibited acetylcholinesterase between compounds. No statistically significant differences were present in the amount of aged acetylcholinesterase between compounds.

activity of the treated groups was significantly different from control levels at all time periods sampled. Over 90% inhibition was observed through 12 h with both compounds, with recovery of activity thereafter (Table 1 and Figure 1). The amount of inhibition was similar in both treatment groups through 1 d (79–81%), but afterward, the recovery of AChE activity in the parathion-treated group was faster than that of the chlorpyrifos-treated group. There were no statistically significant differences between the two groups until d 10, when chlorpyrifos treatment yielded 70% inhibition while parathion treatment yielded only 44%. On d 16, inhibition of AChE in the chlorpyrifos treated group was still 47% compared with only 28% in the parathion group.

Aging of AChE following exposure to the phosphorothionates was very similar between compounds. Following exposure to parathion, the percentage of aged AChE was 25% at 4 h and remained at this level through d 2 (Table 1 and Figure 1). This percentage reached a maximum of 38% at d 4 and decreased to 26% on d 16. Following exposure to chlorpyrifos, 20% of the AChE was aged at 4 h and increased to 34% by d 2. This percentage reached a maximum of 50% at d 8, with slow recovery to 39% on d 16. Although chlorpyrifos resulted in a higher level of aged AChE than parathion did, there were no statistically significant differences in the pattern of aging between the phosphorothionates.

Following in vivo exposure to paraoxon and chlorpyrifos-oxon, maximal inhibition of AChE activity by both compounds occurred at 4–8 h (Table 2

TABLE 2. Specific Activities[a] of Inhibited and Aged Acetylcholinesterase from the Brain of Catfish Exposed to 30 μg/L Paraoxon or 7 μg/L Chlorpyrifos-Oxon In Vivo

Time	Control for vehicle[b]	Control for TMB-4[c]	Chlorpyrifos-oxon		Paraoxon	
			Vehicle	TMB-4	Vehicle	TMB-4
4 h	535.6 ± 50.6^{A}	496.3 ± 45.7^{A}	17.9 ± 4.0^{A}	425.6 ± 26.3^{A}	52.0 ± 21.1^{A}	452.9 ± 37.9^{A}
2 d	499.3 ± 6.5^{A}	459.1 ± 7.2^{A}	193.6 ± 8.9^{B}	337.5 ± 4.2^{B}	214.5 ± 17.5^{B}	335.8 ± 12.3^{B}
4 d	507.2 ± 34.7^{A}	474.9 ± 41.1^{A}	294.7 ± 11.7^{C}	350.6 ± 13.2^{B}	295.8 ± 18.8^{C}	359.6 ± 12.9^{B}
6 d	517.6 ± 57.3^{A}	469.0 ± 24.9^{A}	348.0 ± 7.0^{D}	355.5 ± 11.2^{B}	362.4 ± 6.3^{D}	369.6 ± 1.1^{B}
10 d	612.2 ± 20.3^{A}	507.8 ± 37.5^{A}	439.5 ± 5.6^{E}	406.1 ± 11.8^{B}	464.8 ± 16.4^{E}	437.9 ± 17.9^{AB}
16 d	505.8 ± 29.1^{A}	478.0 ± 23.4^{A}	446.9 ± 40.0^{E}	414.9 ± 34.1^{A}	448.4 ± 34.3^{E}	412.7 ± 26.7^{AB}

[a]Specific activity is expressed as nmol product formed/min-mg protein, mean ± SEM of three catfish. Means within each column not followed by the same capital letter are significantly different ($p \leq .05$). All means from chlorpyrifos-oxon or paraoxon exposures are significantly different from controls ($p \leq .05$) except for 16 d.

[b]Specific activities were obtained from brain homogenates preincubated with the reactivator vehicle. Differences from control specific activities represent amount of inhibited AChE.

[c]Specific activities were obtained from brain homogenates preincubated with the reactivator, TMB-4. Differences from control specific activities represent amount of aged AChE.

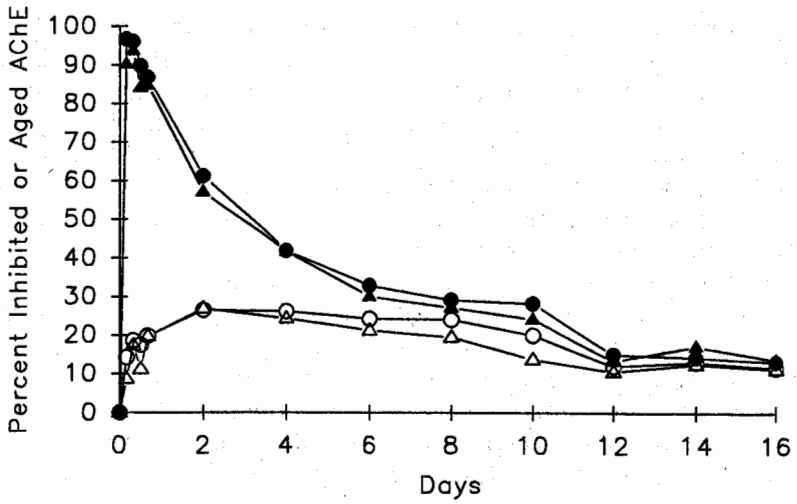

FIGURE 2. Plot of percent inhibited (closed symbols) and aged (open symbols) acetylcholinesterase in catfish brain as a function of time following a 4-h in vivo exposure to 30 μg/L paraoxon (triangles) or 7 μg/L chlorpyrifos-oxon (circles). No statistically significant differences were present in the amount of inhibited or aged acetylcholinesterase between compounds.

and Figure 2). The recovery of AChE activity was initially rapid, with inhibition decreasing to 42% with both compounds by d 4, with somewhat slower recovery afterward. The AChE activity of the treated groups was significantly different from that of control levels until d 12 when only 13–15% inhibition remained. The patterns of recovery of AChE activity were similar between the two oxons with no statistical differences at any sample time.

Catfish brain AChE aged similarly following paraoxon and chlorpyrifos-oxon exposures (Table 2 and Figure 2). Following exposure to paraoxon, initial aged AChE was 9% of control at 4 h, while following chlorpyrifos-oxon, 14% of AChE was aged. From 8 h on, the amount of aged AChE was identical between the compounds with very few differences beyond that time point. Maximum aging occurred by 2 d, with 26–27% aged AChE with both oxons. There were no significant statistical differences in the amount of aged AChE at any time point between compounds.

DISCUSSION AND CONCLUSIONS

The slower return of AChE activity in the chlorpyrifos-exposed catfish compared with the parathion-exposed catfish is most likely influenced by the lipophilicity of the compounds. Chlorpyrifos is much more lipophilic than is parathion, exhibiting hexane:acetonitrile partition coefficients of 0.285 and 0.062, respectively (Chambers & Carr, 1993). Likewise, chlorpyrifos has a much higher hexane:acetonitrile partition coefficient than those of paraoxon, 0.025, and chlorpyrifos-oxon, 0.0625. The hydrophobic characteristics

of chlorpyrifos suggest that once introduced into the water column, the compound is absorbed quickly. It then partitions into the fat stores of the catfish, and within 24 h, the fat contains the highest concentration of chlorpyrifos compared with other tissues (Barron et al., 1993). The partitioning of chlorpyrifos into nonpolar compartments in the fish could lead to its gradual release into the bloodstream and bioactivation, thereby delaying the recovery of AChE by creating the opportunity for further inhibition. The substantially higher partition coefficient of chlorpyrifos compared with its active metabolite suggests that it is the parent compound, not the oxon, that bioaccumulates. A more persistent inhibition of AChE following chlorpyrifos than parathion or either oxon has been previously observed in rats (Chambers & Carr, 1993).

The bioaccumulation of chlorpyrifos in catfish tissue is strongly suggested by the more persistent inhibition of AChE following chlorpyrifos exposure compared with the other compounds tested. It is unlikely that further chlorpyrifos absorption from the aquaria was occurring because the flow rate allowed for total exchange of the water in 1 h, thereby removing chlorpyrifos from the aquaria. Therefore, in the late sampling periods, very little chlorpyrifos was expected to be available for uptake.

The greater toxicity of chlorpyrifos compared with parathion could also be related to the lipophilicity of the compounds, with chlorpyrifos expected to display the higher uptake by the gill. It has been demonstrated that uptake of OP insecticides by fish is directly related to their octanol:water partition coefficients and that their elimination is inversely related to their coefficients (de Bruijn & Hermens, 1991; de Bruijn et al., 1993). The faster uptake of chlorpyrifos (log K_{ow} of 5.11) compared with parathion (log K_{ow} of 3.81) (Verschueren, 1983) would allow the former to be more available for rapid metabolism to its active metabolite and interaction with its target.

The chlorpyrifos concentration used here to obtain greater than 90% AChE inhibition was 10-fold lower than that used for parathion. However, the chlorpyrifos-oxon concentration used to obtain greater than 90% AChE inhibition was only fourfold lower than that used for paraoxon. This smaller relative difference between the concentrations of the two oxons than between the concentrations of the two phosphorothionates suggests that, once the lipophilic characteristics of chlorpyrifos are reduced, the toxicities of the two compounds are more similar. The higher toxicity of chlorpyrifos-oxon compared with paraoxon is best attributed to the former's greater potency as an inhibitor of catfish AChE from brain and other tissues (Straus & Chambers, 1992). Another possible factor that could play a role in the toxicity differences between parathion and chlorpyrifos is differences in biotransformation, which is not well characterized in catfish at present.

Aging of phosphorylated AChE in catfish exposed to OP insecticides is dependent on the amount of inhibition and the persistence of that inhibition. Although not significant statistically, chlorpyrifos exposure yielded more aged AChE than did parathion. This higher level of aged enzyme seems to be related directly to the more persistent inhibition observed following chlor-

pyrifos exposure than that following parathion exposure. The more persistent the inhibition of AChE, the greater the opportunity for aging of the enzyme to occur.

The mechanism behind the persistent inhibition of AChE in catfish following exposure to phosphorothionate insecticides is not the rapid aging of all of the AChE. This differs from the persistent inhibition of AChE observed in rats exposed to phosphorothionate insecticides (Carr & Chambers, 1992), where rapid recovery of AChE activity via spontaneous reactivation occurs until all the inhibited AChE is aged, about 4 d postexposure with parathion and 10 d with chlorpyrifos. However, in catfish, at 10 d postexposure there was still inhibited AChE present that was not aged. Additionally, following exposure to the active metabolites, comparison with the aging of AChE in rat brain (unpublished results) suggests that the rate of spontaneous reactivation of inhibited AChE is a very slow process in catfish compared to rat. By 3 d postexposure all the inhibited AChE in rat brain was aged, but a similar situation did not occur until 12 d postexposure in catfish brain. Therefore, a slower rate of reactivation could contribute substantially more than aging to the persistent inhibition observed in exposed catfish. A lack of spontaneous reactivation of brain AChE has been observed during in vitro experiments with rainbow trout (Wallace & Herzberg, 1988).

In summary, aging of inhibited AChE did not appear to contribute greatly to the persistent inhibition of AChE in channel catfish exposed to OP insecticides. The more persistent AChE inhibition following chlorpyrifos exposure than parathion exposure was probably a result of the greater lipophilicity of the former compound. Additionally, the greater toxicity of chlorpyrifos than parathion to catfish seems to be directly related to its lipophilicity also.

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